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(54) Transgenic animals secreting desired proteins into milk.

(57) A DNA sequence contains a gene encoding a protein, the gene being under the transcriptional control in the DNA sequence of a mammalian milk protein promoter which does not naturally control the transcription of the gene, such DNA sequence including DNA enabling secretion of the protein.

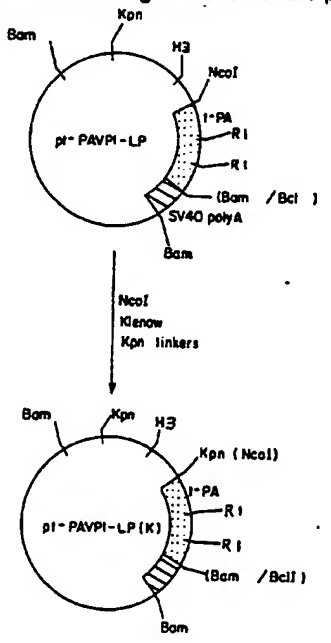

FIG 1

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TRANSGENIC ANIMALS SECRETING DESIRED PROTEINS INTO MILK

This invention relates to transgenic animals.

It is possible to insert foreign genes into vertebrate embryos, and for these genes to be incorporated into the genome of the resulting animal. Insertion of the foreign genes has been carried out mechanically (by microinjection), and with the aid of retrovirus vectors (for example, as is described in Huszar et al. (1985) P.N.A.S. U.S.A 82 , 8587). The animals resulting from this process are termed "transgenic." The foreign genes can be sexually transmitted through subsequent generations and are frequently expressed in the animal. In some instances the proteins encoded by the foreign genes are expressed in specific tissues. For example, the metallothionein promoter has been used to direct the expression of the rat growth hormone gene in the liver tissue of transgenic mice (Palmiter et al., 1982 Nature 300:611). Another example is the elastase promoter, which has been shown to direct the expression of foreign genes in the pancreas (Ornitz et al., 1985 Nature 313:600). Developmental control of gene expression has also been achieved in transgenic animals, i.e., the foreign gene is transcribed only during a certain time period, and only in a particular tissue. For example, Magram et al. (1985, Nature 315:338) demonstrated developmental control of genes under the direction of a globin promoter; and Krumlauf et al. (1985, Mol. Cell. Biol. 5:1639) demonstrated similar results using the alpha-feto protein minigene.

In a first aspect thereof, the invention features a DNA sequence containing a gene encoding a protein, the gene being under the transcriptional control of a mammalian milk protein promoter which does not naturally control the transcription of the gene, the DNA sequence further including DNA enabling secretion of the protein; e.g., a secretion signal-encoding sequence interposed between the gene and promoter. The promoter can be that of a milk serum protein or a casein protein, although milk serum proteins are preferred, as will be discussed in more detail below. (As used herein, "gene" refers to both genomic DNA sequences and cDNA sequences.)

The invention permits the production of any desired protein in an easily maintained stable, portable culture system, i.e., a living domesticated mammal, which is capable not only of producing the desired protein, but preferably of passing on the ability to do so to its female offspring as well. Secretion of the protein into the host mammal's milk facilitates purification and obviates removal of blood products and culture media additives, some of which can be toxic or carcinogenic. More importantly, protein yields will be high and production will be more cost effective and efficient.

The invention thus extends to a mammalian embryo having a nucleus containing a DNA sequence according to this invention.

According to a second and alternative aspect of this invention, we provide a mammal in which the genome of the mammary glands of said mammal comprises a gene encoding a protein, said gene being under the transcriptional control of a mammalian milk protein promoter which does not naturally control the transcription of said gene, said genome comprising DNA enabling the secretion of said gene encoding said protein.

The invention provides, in a third and further alternative aspect thereof, a method for producing a protein comprising the steps of:

(a) inserting into a mammalian embryo a DNA sequence comprising a gene encoding said protein, said gene being under the transcriptional control of a milk protein promoter which does not naturally control the transcription of said gene, said DNA sequence comprising DNA enabling secretion of said protein,

(b) allowing said embryo to develop into an adult mammal,

(c) inducing lactation in said mammal, or in a female descendant of said mammal in which said gene, promoter, and signal sequence are present in the mammary tissue genome,

(d) collecting milk of said lactating mammal, and

(e) isolating said protein from said collected milk.

The invention is hereinafter more particularly described by way of example only with reference to the accompanying drawings, in which:-

Fig. 1 is a diagrammatic representation of the construction of an embodiment of intermediate vector in accordance with the present invention, namely pt-PA VPI-LP(K);

Fig. 2 is a diagrammatic representation of the construction of another embodiment of intermediate vector in accordance with the invention, namely pWAP (H_3);

Fig. 3 is a diagrammatic representation of the construction of another vector in accordance with the invention, namely pWAP-t-PA(S);-

Fig. 4 is a diagrammatic representation of the construction of another intermediate vector in accordance with the invention, namely pHbsSVA; and

Fig. 5 is a diagrammatic representation of the construction of another vector according to the invention, namely pWAP-Hbs(S).

DNA Sequence Elements

Promoter

The milk protein promoter can be derived from any mammalian species, and can be any promoter naturally associated with any protein which is normally secreted into mammalian milk. Generally, milk proteins are classified as the caseins, which are defined herein as the milk proteins which are present in milk in the form of micelles, and which are removed from skim milk by clotting with rennet; and the milk serum proteins, which are defined herein as the non-casein milk proteins. Whey proteins constitute the predominant fraction of the milk serum proteins, and in rodents include the protein known as whey acid protein. Whey acid protein ("WAP") is named based on its acidic isoelectric point (Piletz (1981) J. Biol. Chem. 256: 11509). Another example of a milk serum protein described in the literature is α-lactalbumin (described, along with mouse WAP, in Hennighausen and Sippel (1982) Eur. J. Biochem. 125, 131). Milk proteins are discussed in detail in Walstra and Jenness Dairy Chemistry and Physics (John Wiley & Sons 1984).

Generally, milk serum protein promoters are preferable to casein promoters in the practice of the present invention
because caseins generally are produced in female mammals during pregnancy as well as after birth, while WAP is expressed primarily during post-partum lactation. This difference is of potential importance for two reasons. First, pre-birth production of the desired protein under the transcriptional control of a casein promoter could be wasteful, since the protein cannot be isolated from milk until it is secreted into the milk post-partum. Second, where the desired protein is toxic in large amounts (human tissue plasminogen activator (t-PA) is an example), a build-up of the protein in the tissues prior to lactation could be deleterious to the health of the host mammal. An additional advantage of some whey promoters such as the WAP promoter is that they are strong promoters, as evidenced by the large amounts of some whey proteins present in milk. Casein promoters also have this advantage.

Milk protein genes from which promoters, in addition to the WAP promoters, can be isolated, can be obtained in the same manner in which the WAP genes were isolated, as described in Hennighausen and Sippel, id, and Campbell et al. (1984) Nucleic Acids Research 12, 8685. The method generally involves isolating the mRNA from a lactating mammary gland, constructing a cDNA library from the mRNA, screening the library for the particular milk protein cDNA being sought, cloning that cDNA into vectors, and using the appropriate cDNA as a probe to isolate the genomic clone from a genomic library. A sequence upstream from the transcription start site in the genomic clone constitutes a putative "promoter", a genomic sequence preceeding the gene of interest and presumed to be involved in its regulation. The promoter may be isolated by carrying out restriction endonuclease digestions and subcloning steps. Promoters need not be of any particular length nor to have directly shown any properties of regulation. The mouse WAP promoter was isolated as a 2.6 kb EcoRI - KpnI fragment immediately 5' to the WAP signal sequence.

Desired Protein

Any desired protein can be produced according to the invention. Preferred proteins are proteins useful in the treatment, prevention, and/or diagnosis of human disease; examples are t-PA and hepatitis B surface antigen. The invention is particularly useful for proteins which must be produced on a large scale to be economical, e.g., industrial enzymes and animal proteins.

Signal Sequence

It is necessary, for secretion of the desired protein into the milk of the host mammal, that the DNA sequence containing the gene for the desired protein include DNA which, when translated, causes the protein to be secreted out of the mammary tissue into the milk. Without such a sequence, the desired protein would remain in the mammary tissue, from which purification would be difficult, and would require sacrifice of the host animal. This DNA can ncode a hydrophobic secretion signal which is cleaved during

secretion. The signal sequence can be that which is naturally associated with the desired protein, if the protein is normally secr ted (e.g., t-PA). Alternatively, the signal encoding sequence can be that of the milk protein providing the promoter, i.e., when the milk protein gene is digested and the promoter isolated, a DNA fragment is selected which includes both the promoter and the signal encoding sequence directly downstream from the promoter. Another alternative is to employ a signal ncoding sequence derived from another secreted protein, which is neither the milk protein normally xpressed from the promoter nor the desired protein.

Termination Site

Preferably there is located within or downstream from the 3' end of the desired gene a termination site. This site may be provided by sequences in the gene itself, or may need to be added. If the sequence is to be added, a preferred sequence is provided by the polyadenylation sequence of the virus SV40, as will be described in greater detail below.

Methods

Genetic Manipulations

Generally, all DNA manipulations used in the genetic constructions described herein may be carried out using conventional techniques, as described, e.g., in Maniatis et al. Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, 1982).

Introduction of DNA into Embryos

Once the genetic constructions have been produced in vectors, e.g., plasmids, the promoter-signal sequence-desired protein-termination sequence DNA fragment is excised and then introduced into the desired mammalian embryo using, e.g., retroviruses or standard microinjection methods such as are described in Kraemer et al. (1985), Costantini and Jaenisch, eds., Genetic Manipulaton of the Early Mammalian Embryo, Cold Spring Harbor Laboratory (bovine embryo microinjection); Hammer et al. (1985) Nature 315, 680 (rabbit, sheep, and porcine embryo microinjection); and Gordon and Ruddle (1984) Methods in Embryology 101, 411 (mouse embryo microinjection). Micro-injection is preferably carried out on an embryo at the one-cell stage, to maximize both the chances that the injected DNA will be incorporated into all cells of the animal, including mammary tissue, and that the DNA will also be incorporated into the germ cells, so that the animal's offspring will be transgenic as well. Microinjection is a standard technique which involves, briefly, isolating fertilized ova, visualizing the pronucleus, and then injecting the DNA into the pronucleus by holding the ova with a blunt holding pipette of a diameter on the order of 50 μm, and using a sharply pointed pipette of a diameter on the order of 1.5 μm to inject buffer-containing DNA into the pronucleus. Following microinjection, the transgenic female animals are allowed to become sexually mature, mated, and milk collected post-partum.

Preferred host mammals are those which are already bred for large volume milk production, e.g., cows, sheep, goats, and pigs

t-PA Production

There will now be described the construction of plasmid DNA in which the gene encoding human uterine t-PA, including the signal encoding sequence, is under the transcriptional control of the mouse WAP promoter, and has at its 3' end the SV40 polyadenylation site. This DNA was made from two intermediate plasmids, one carrying the mouse WAP promoter and one carrying the t-PA signal and structural sequences, as well as the SV40 polyadenylation site.

The WAP promoter containing plasmid pWAP-CAT (Fig. 2, obtained from Lothar Hennighausen, National Institutes of Health) was derived from a plasmid made according to the methods described in Hennighausen and Sippel (1982) Eur. J. Biochem. 125, 131; and Campbell et al. (1984) Nucleic Acids Research 12, 8685. In addition to containing the mouse WAP promoter, pWAP-CAT contains a gene which, for pr sent purposes, is irrelevant: the CAT (chloramphenical acetyltransferase) gene, which does not form a part of the final DNA sequence which is microinjected.

Still referring to Fig. 2, pWAP-CAT was modified to convert the Eco RI site to a HindIII site using Klenow and Hind III linkers.

The t-PA-containing plasmid pt-PA-VPI-LP(K) (Fig. 1) was derived from pt-PAVPI-LP, containing the t-PA gene (including the t-PA signal encoding sequence) and SV40 polyadenylation site, by modifying the unique NcoI site at the 5'end of the t-PA gene using NcoI endonuclease and Klenow and adding Kpn linkers to produce a KpnI site.

Referring to Fig. 3, the KpnI-BamHI fragment of pt-PA VPI-LP(K), containing the t-PA gene and SV40 sequences, was isolated and ligated to BamHI-KpnI treated pWAP(H3) to form pWAP-tPA (S), which was then transformed into a TET-sensitive derivative of E. coli strain MC1061. This transformed strain, containing plasmid DNA in which the HindIII-BamHI fragment contains the t-PA gene including the t-PA signal encoding sequence under the transcriptional control of the WAP promoter and followed by the SV40 polyadenylation site, has been deposited in the American Type Culture Collection on March 13, 1986 and given ATCC Accession No. 67032.

Production of milk into which t-PA has been secreted is carried out by excising the HindIII-BamHI fragment from the deposited strain and transferring it by microinjection or other means preferably into the one-cell embryo of a mammal according to conventional methods, as described above. Alternatively, though less desirably, the entire plasmid or other restriction fragments can be introduced into the embryos. Embryos are then nurtured to term in vivo. Animals born from such manipulated embryos are screened for the presence of introduced DNA in the genome, and expression of t-PA in the milk is screened for among transgenic, lactating females. The protein from the milk of the adult lactating female animal will be assayed for t-PA by conventional procedures.

Production of Hepatitis B Surface Antigen

Referring to Figure 5, intermediate vectors pWAP-CAT and pHBsSVA were used to construct pWAP-Hbs(S), containing the gene for hepatitis B surface antigen, under the transcriptional control of the WAP promoter and followed by the SV40 polyadenylation site.

The plasmid pWAP-CAT is described above. Plasmid pHbsSVA was constructed as illustrated in Fig. 4. $pCLH_3A$, containing the SV40 polyadenylation sequence, was restricted with Eco RI, SacI, and BglII. pBSBam, containing the gene for hepatitis B surface antigen, was cut with EcoRI, BamHI and PvuI, and the two mixtures ligated to give pHbsSVA, in which the SV40 sequence was positioned at the 3' end of the Hbs gene, on a BamHI-BglII fragment. This fragment was then ligated (Fig. 5) to BamHI and bacterial alkaline phosphatase-treated pWAP-CAT, transformed into E . coli strain MC1061, and the plasmid pWAP-Hbs(S) isolated.

The BamHI-EcoRI fragment of WAP-Hbs(S) can be excised and used as described above to produce hepatitis B surface antigen. Alternatively, though less desirably, the entire plasmid or other restriction fragments can be introduced into the embryos. Embryos are then nurtured to term in vivo. Animals born from such manipulated embryos are screened for the presence of introduced DNA in the genome, and expression of hepatitis B surface antigen in the milk is screened for among transgenic, lactating females. pWAP-Hbs(S) has been deposited in the American Type Culture Collection on March 13, 1986 and given ATCC Accession No. 67033. Applicants' assignee, Integrated Genetics, Inc.,

Both pWAP-Hbs(S) and pWAP-t-PA(S) can be used as cassette vectors in which the hepatitis B surface antigen gene or the t-PA gene can be excised and replaced, using conventional methods, with any desired gene. If desired, the signal encoding sequence from pWAP-t-PA(S) can be left in the vector, and a gene lacking such a sequence inserted downstream of and in frame with it. Alternatively, the signal sequence from pWAP-t-PA(S) or pWAP-Hbs(S) can be removed along with the structural gene and the signal encoding sequence of the substituted gene employed. In addition, the WAP promoter alone can be excised and inserted into another desired expression vector.

Purification and Use

The proteins produced by a process according to the invention are purified from the milk into which they have been secreted and used for their known purposes.

Hepatitis B surfac antigen is useful in the production of hepatitis B vaccine.

t-PA is useful in the treatment of thrombolytic disease in which fibrin clot lysis is necessary, as described in European Patent Application 85306957.3. That Patent Application also describes general purification techniques which will be useful for milk-secreted proteins.

Stability in Milk

Table I below shows that, despite the presence in milk of numerous proteases, recombinant t-PA is stable when added to raw goat milk and incubated at 20° or 37°C for 24 hours, with no evidence of loss of activity, as measured using the standard fibrin plate test (results not shown in Table I) or the amidolytic assay described in Wei et al., id. Similarly, recombinant hepatitis B surface antigen was found to be stable for at least 24 hours in raw goat milk (data not shown).

Table I

Amidolytic assay for TPA

	Incubation Time	Temperature	Units/ml
Goat milk alone	--	--	<20, <20
Goat milk & TPA	0	--	437, 368
Goat milk & TPA	24 hours	20°C	419, 434
Goat milk & TPA	24 hours.	37°C	467, 507

Other Embodiments

Other embodiments are feasible. For example, other milk serum protein promoters can be used in place of the mouse WAP promoter, and the promoter can be derived from any mammalian species. For example, milk serum protein promoters such as that of β-lactoglobulin can be used, and the rat, rather than mouse, WAP promoter can be used; the rat WAP promoter is described in Campbell et al., id. Although less desirable than milk serum protein promoters, casein promoters can be used as well. The protein produced using processes in accordance with this invention can be any desired protein of therapeutic or industrial importance.

Claims

I. A DNA sequence containing a gene encoding a protein, said gene being under the transcriptional control in said DNA sequence of a mammalian milk protein promoter which does not naturally control the transcription of said gene, said DNA sequence further comprising DNA enabling secretion of said protein.

2. The DNA sequence of claim I, wherein said secretion-enabling DNA comprises a secretion signal-encoding sequence interposed between said gene and said promoter.

3. The DNA sequence of claim I wherein said milk protein is a milk serum protein or a casein protein.

4. The DNA sequence of claim 3 wherein said milk serum protein is a whey acid protein.

5. The DNA sequence of claim I wherein said signal encoding sequence is the signal encoding sequence naturally associated with said gene encoding said protein.

6. The DNA sequence of claim I wherein said signal encoding sequence is the signal encoding sequence naturally associated with said mammalian milk protein promoter.

7. The DNA sequence of claim I wherein said DNA sequ nce includes a transcriptional stop sequence.

8. The DNA sequence of claim 7 wherein said stop sequenc is derived from SV40 virus DNA.

9. The DNA sequence of claim 7 wherein said stop sequence is contained in the polyad nylation sequence of SV40.

10. A mammalian embryo having a nucleus containing the DNA sequence of claim 1.

11. The DNA sequence of claim 1 wherein said protein is human tissue plasminogen activator or hepatitis B surface antigen.

12. A mammal in which the genome of the mammary glands of said mammal comprises a gene encoding a protein, said gene being under the transcriptional control of a mammalian milk protein promoter which does not naturally control the transcription of said gene, said genome comprising DNA enabling the secretion of said gene encoding said protein.

13. The mammal of claim 11, said mammal being a sheep, pig, goat, cow, or other mammals.

14. The mammal of claim 11 wherein said gene is expressed in greater amounts during lactation than during pregnancy.

15. A method for producing a protein comprising the steps of:

(a) inserting into a mammalian embryo a DNA sequence comprising a gene encoding said protein, said gene being under the transcriptional control of a milk protein promoter which does not naturally control the transcription of said gene, said DNA sequence comprising DNA enabling secretion of said protein,

(b) allowing said embryo to develop into an adult mammal,

(c) inducing lactation in said mammal, or in a female descendant of said mammal in which said gene, promoter, and signal sequence are present in the mammary tissue genome,

(d) collecting milk of said lactating mammal, and

(e) isolating said protein from said collected milk.

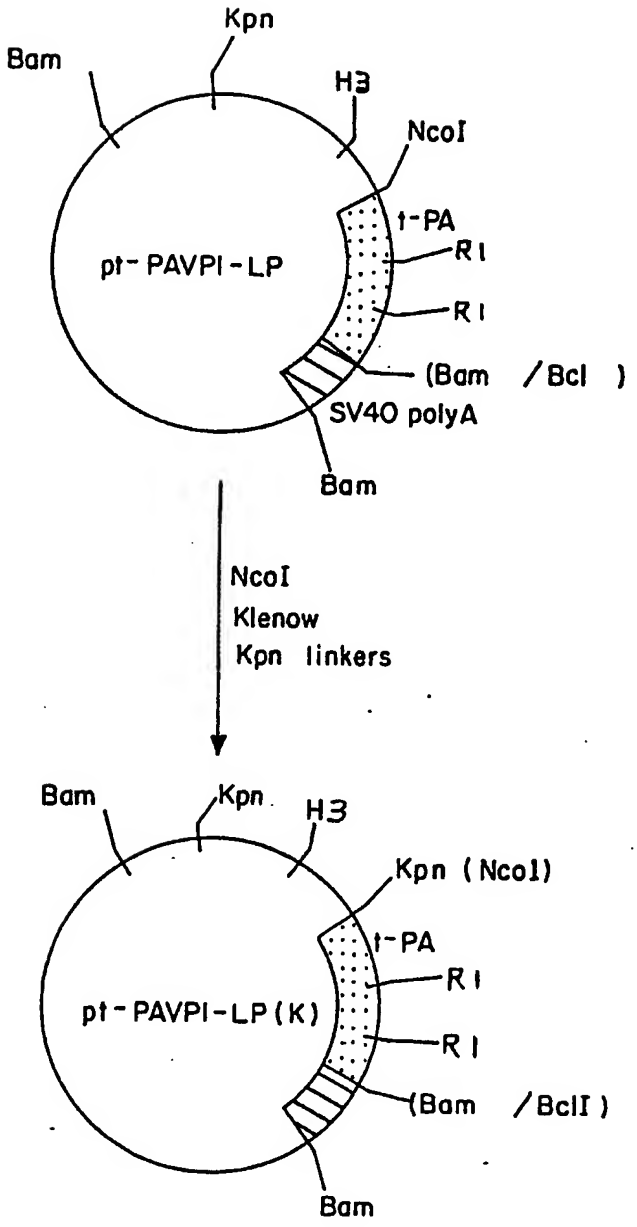

FIG 1

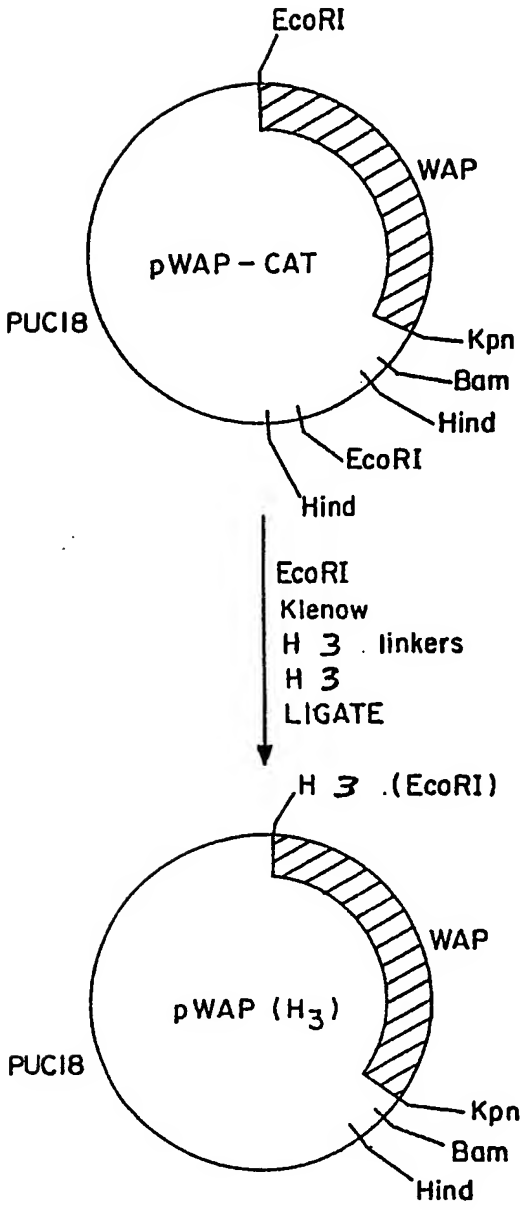

FIG 2

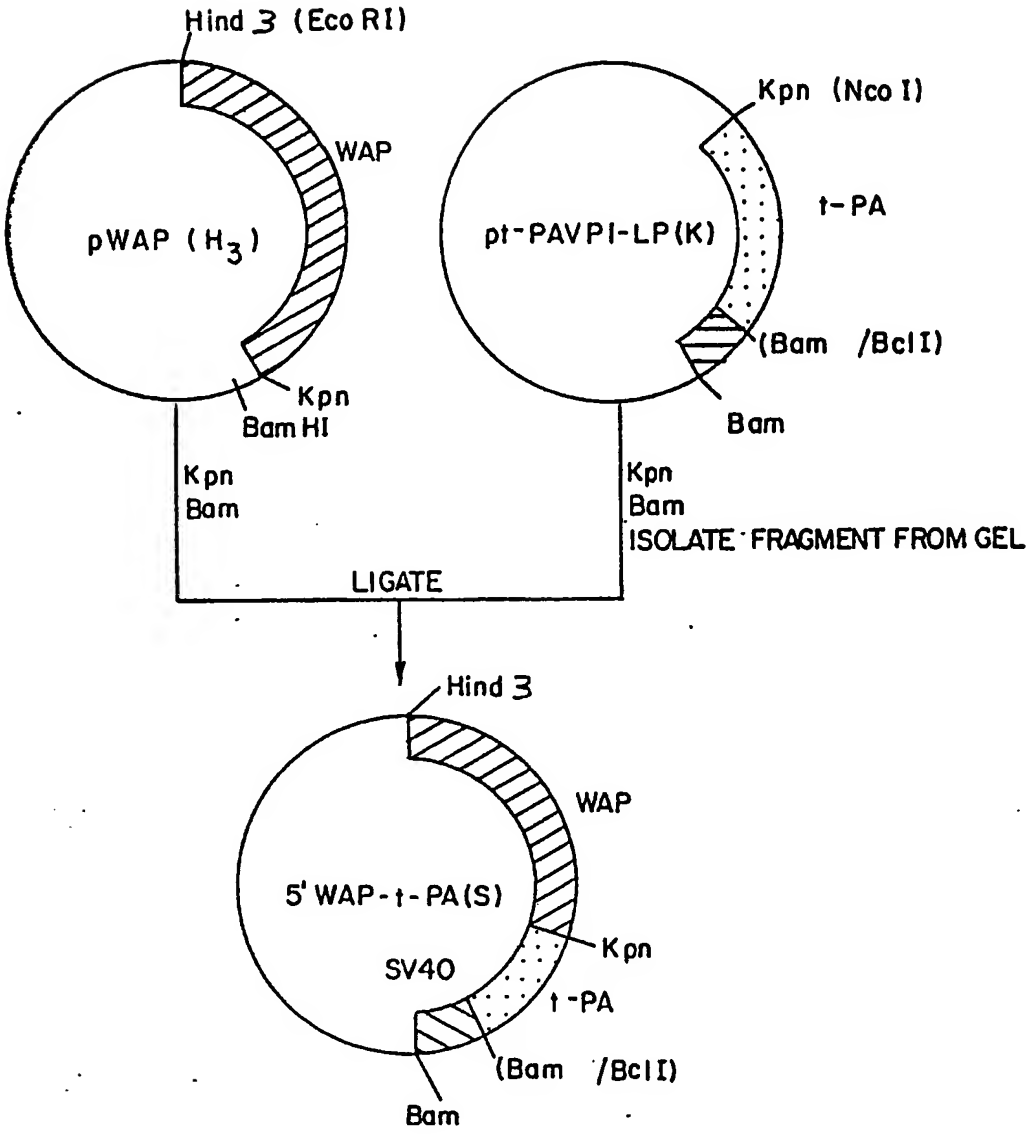

FIG 3

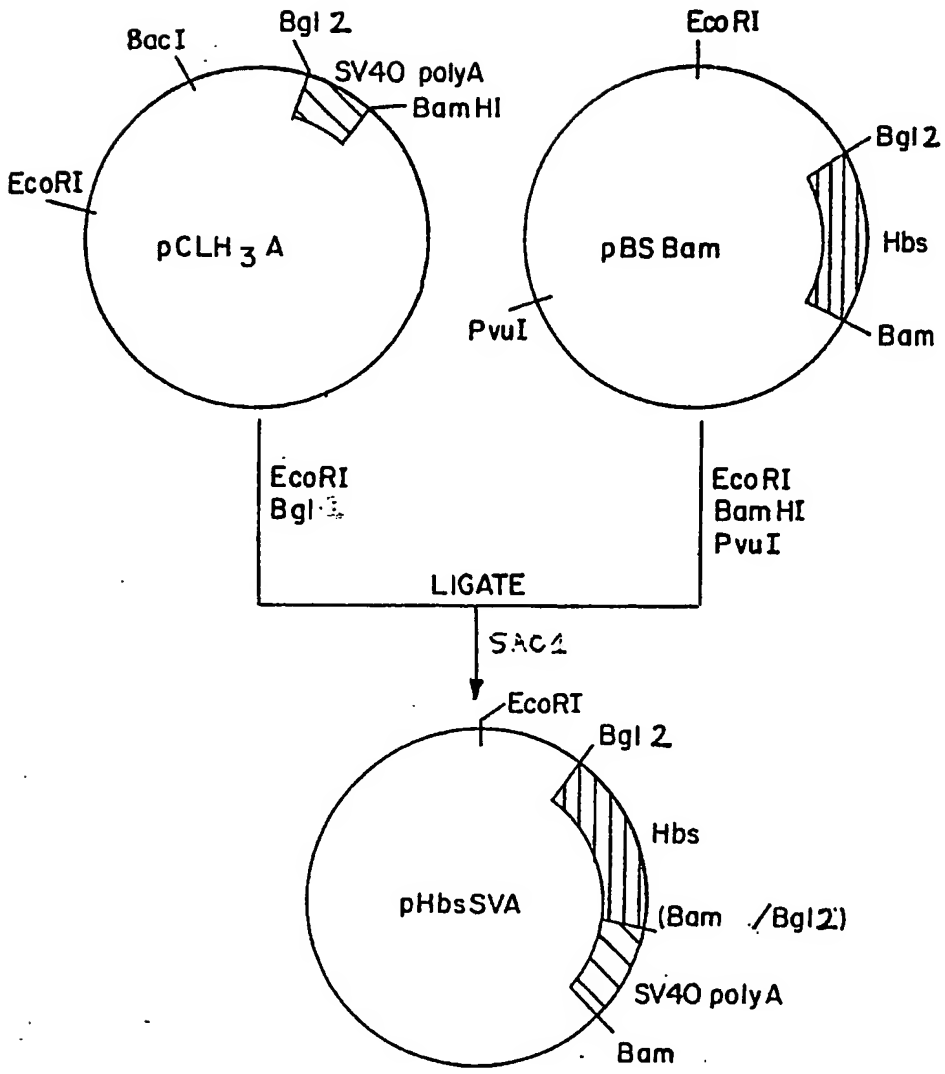

FIG 4

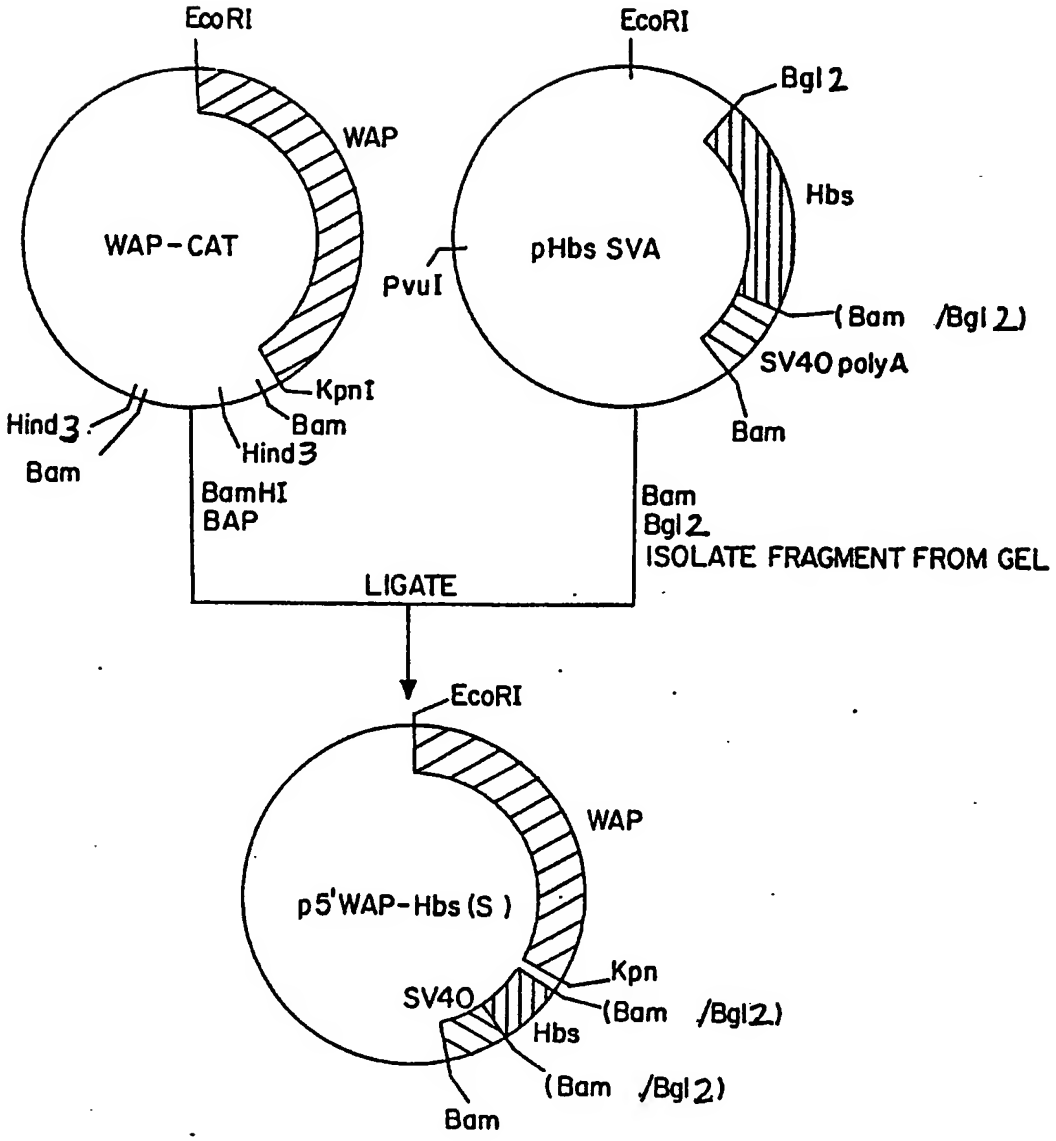

FIG 5

European Patent Office

EUROPEAN SEARCH REPORT

Application number

DOCUMENTS CONSIDERED TO BE RELEVANT

EP 87303112.4

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	EP - A1 - 0 169 672 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) * Claims 1,4,13 * --	1,10, 12,15	C 12 N 15/00 C 07 H 21/04 C 12 P 21/00
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D,A	NATURE, vol. 315, May 23, 1985, New York, London J. MAGRAM et al. "Developmental regulation of cloned adult β-globin gene in transgenic mice" pages 338-340 * Totality * --	10,12	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 N C 07 H C 12 P
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	25-06-1987	WOLF

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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D : document cited in the application
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EUROPEAN SEARCH REPORT

Application number

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
D,A	MOLECULAR AND CELLULAR BIOLOGY, vol 5, no. 7, July 1985, Washington DC R. KRUMLAUF et al. Developmental Regulation of α-Fetoprotein Genes in Transgenic Mice" pages 1639-1648 * Totality * --	10,12	
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			TECHNICAL FIELDS SEARCHED (Int. Cl.4)

The present search report has been drawn up for all claims

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